Europäisches Patentamt
European Patent Office
Office européen des brevets

Bescheinigung Certificate Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

02078624.0

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

Anmeldung Nr:
Application no.: 02078624.0
Demande no:

Anmeldetag:
Date of filing: 04.09.02
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

DSM N.V.
Het Overloon 1
6411 TE Heerlen
PAYS-BAS

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

A nutritional and therapeutic composition

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

A61K38/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR IE IT LI LU MC NL PT SE SK TR

A NUTRITIONAL AND THERAPEUTIC COMPOSITION

Field of the invention

The present invention relates to a composition comprising an insulin sensitizer.

Background of the invention

Type 2 diabetes mellitus is a clinically and genetically heterogeneous group of syndromes characterized by elevated blood glucose levels. It occurs because the insulin produced by the b (beta) cells of the pancreas is either insufficient or ineffectively utilized by target tissues, resulting in high levels of glucose in the blood. Post-prandial peaks (a rise of glucose in the body after a meal), which usually result from a high carbohydrate diet, contribute to the high blood glucose levels. Complications that surface from diabetes can usually be traced back to excessive sugar levels in the blood over a period of many years.

The progression of type 2 diabetes is often characterised as follows. At first a slow but progressive increase in insulin resistance develops. This implies that insulin production by pancreas is normal during the early stages of the disease, but the ability of insulin to increase glucose uptake is reduced. The reduced insulin sensitivity in the early stages of the disease is usually compensated by an increased release of insulin by the pancreas. However, after 5-10 years the pancreas does not longer properly respond to glucose ingestion and the reduced insulin sensitivity is accompanied by a suboptimal insulin production. The resulting hyperglycemea (= high blood glucose levels) leads to a rapid disabilitating progression in the disease process and the necessity to start using of oral medication and finally exogenous insulin therapy.

The abnormal high levels of glucose in the blood may contribute to various micro and macrovascular complications, including cardiovascular disease, retinopathy, nephropathy and neuropathy. Serious health complications resulting from these high glucose levels, include eye, heart, kidney, and nerve damage.

The key to fighting diabetes is through monitoring and controlling blood sugar levels. If a patient conscientiously monitors and controls his blood sugar levels at an early stage, he may delay or prevent many complications associated with the disorder. A proper diet and exercise can help people with diabetes to maintain healthy blood glucose levels. However, when diet and exercise are inadequate to control diabetes, medication is required. At this stage treatment can still rely on the use of oral anti-diabeties drugs alone, i.e. exogenous insulin application is not yet necessary.

In principle three classes of oral anti-diabetes drugs are available, i.e. the "blocker" class, the "stimulator" class and the "sensitizer" class. The "blocker" class of oral anti-diabetes agents has been shown to delay or prevent further development of the disease. Examples of this class of agents are the so-called alpha-glucosidase inhibitors, which act by delaying the absorption of glucose from the ingested carbohydrates. The "stimulator" class of oral anti-diabetes agents stimulate the production of insulin by the pancreas. Examples of the "stimulator" class are sulfonylureas, which are known to be effective to stimulate secretion of insulin. The "sensitizer" class of oral anti-diabetes agents help to use glucose more efficiently or to make tissue cells more sensitive to insulin. Examples of medical "insulin sensitizers" are biguanides (such as Glucophage) and thiazolidinediones (such as Actos and Avandia). Collectively the latter products are usually referred to as "insulin sensitizers". By "insulin sensitizer" or "insulin sensitzing agent" is meant a compound that will lower blood glucose levels by increasing the responsiveness of the tissues to insulin.
Not surprisingly several of these oral anti-diabetic drugs may have undesirable side effects. For example, adverse effects of biguanide antidiabetic agents include gastro-intestinal disturbances and lactic acidoses. Adverse effects of sulphonylurea antidiabetic agents include hypoglycaemia, gastro-intestinal disturbances and hypersensitivity reactions.

Apart from these oral anti-diabetes drugs, a number of natural compounds known for their insulin sensitising effect are being used. The obvious advantage of the latter group of compounds, which includes various trace elements and vitamins, is that they may form part of the regular human diet already. One of these natural insulin sensitizers is chromium. Chromium is a trace mineral that's essential for normal insulin function. Dietary studies

indicate that most people in the U.S. and other industrialized countries don't get enough chromium, and deficiencies appear to be even more common in diabetic people. Many clinical studies support the benefits and safety of chromium supplementation in diabetic people. Supplemental chromium is known to lower blood insulin levels, improve glucose tolerance and decrease haemoglobin glycosylation in people with type 2 diabetes. Chromium also helps maintain healthy blood lipid levels, in particular triglycerides and HDL cholesterol. Experts, such as Richard Anderson, Ph.D. from the U.S. Dept. of Agriculture at the Beltsville Human Nutrition Research Center, recommend chromium supplementation in daily amounts of 200-1000 micrograms. Clinical studies show that in particular the organic or chelated forms, such as chromium polynicotinate, picolinate, glycine-niacin chelate, GTF chromium and chromium yeast are effective. Furthermore it has been shown that combinations of chromium and biotin (i.e. Vitamine B8) provide a greater than additive effect.

Vanadium is another trace element involved in promoting normal insulin function. Safe and adequate dietary intakes range from approximately 10-100 micrograms of vanadium per day. Some studies have shown that mega-doses of vanadium as vanadyl sulfate can improve glucose tolerance in type 2 diabetics. However, the vanadium doses used in these studies are 200 to 1,000 times above safe dietary intakes, and the long-term safety of these pharmacological doses has not been established. High-dose vanadyl sulfate (i.e., above 100 micrograms/day) should only be taken under medical supervision. Supplementation with up to 100 micrograms/day is safe, and will cover the body's nutritional vanadium needs including the proposed functions of vanadium in insulin and glucose metabolism.

Niacin is another B-vitamin of special importance to diabetics, because 100milligrams/day of niacin has been shown to improve glucose tolerance and fasting blood glucose in diabetics when co-supplemented with chromium.

Banaba Leaf Extract is a newcomer among standardized diabetes herbs and is currently sold under the trade name "Glucosol." The active marker compound in Glucosol, corosolic acid, has been shown to promote the transport of blood glucose into cells. Glucosol is standardized to provide a minimum of 1% corosolic add. The extract was found

to be well-tolerated and safe in human and animal studies. Compared to other herbal extracts used be people with diabetes, Glucosol offers the advantage that it can lower blood glucose in diabetic people without causing hypoglycemia. Also, the clinically effective dose is only 32-48milligrams daily. A series of recent and still unpublished clinical studies conducted in 1999 by Dr. W. Judy at the Southeastern Institute of Biomedical Research (Bradenton, FL), showed significant benefits of Glucosol when taken daily for 30 days at 32 and 48 milligrams per day. One of these studies was a randomized, double-blind crossover study with 12 diabetic subjects taking 48milligrams of the extract. Glucosol lowered fasting blood glucose in people with type 2 diabetes, and the effect was sustained for several weeks even after discontinuation of the supplement. Study reports are available from the manufacturer (Soft Gel Technologies, Inc.) at their website (www.glucosol.com/glucosol/default.htm).

Insulin sensitizers have been shown to be effective in lowering blood glucose levels by increasing the responsiveness of target tissues to insulin. However, as the disease progresses, type 2 diabetic patients gradually loose their ability to produce sufficient insulin. This decrease in insulin production slowly diminishes the effectiveness of the insulin sensitisers so that at a certain point, these people are forced to use even less appreciated pharmaceutical alternatives like insulin injections to lower their levels of blood glucose.

To prevent or postpone this need for a insulin injections or the use of oral pharmaceutical sensitisers of diabetes type 2, there is a need for a composition thatresults in an effective lowering of blood glucose.

Summary of the invention

The present invention provides a composition rich in di-and tripeptides in combination with an insulin sensitizer. This composition is very suitable as diabetic pharmaceutical or food or a food supplement. Especially this composition is useful for Type 2 diabetes. A composition comprising di and tripeptides is found to be useful to treat Type 2 diabetes.

Detailed description of the invention

This invention pertains to compositions for the oral treatment of diabetes mellitus by administering a peptide fraction along with preferably one or more insulin sensitizer agents to lower the levels of glucose in the blood and to methods for preparing the same. The beneficial effect of the composition according to the invention is not limited to individuals suffering from type 2 diabetes but is also recorded for healthy persons so that it can be applied in the prevention of diabetes. Moreover the composition according to the invention is useful to enhance the recovery of healthy people after physical exercise.
The composition can form part of a food, a beverage or a supplement so that it can be used in the manufacture of a large variety of products including dietetic products, shakes, dietary supplements, infant nutrition, beverages such as sports drinks and soft drinks, or various food products or fermented products

The fact that protein intake has a stimulatory role on plasma insulin levels is not new but was already reported in the 1960 's. Later work has identified free amino acids and especially free arginine, leucine, tyrosine and phenylalanine to have strong insulinotropic effects upon their intravenous injection. Very recently experiments have demonstrated the in-vivo insulinotropic potential of free amino acids in combination with protein hydrolysates and carbohydrate upon oral uptake (Van Loon et al, American Journal of Clinical Nutrition, 72:96-105, 2000).

At first sight type 2 diabetic patients are expected to benefit from this stimulated insulin secretion as brought about by the combined intake of free amino acids, protein hydrolysates and carbohydrates. We have found that peptides and free amino acids do stimulate the insulin response. Howeve, this enhanced insulin production did not result in significant lower blood glucose levels in late stage type 2 diabetic patients. Only the combination of a peptide fraction with a suitable insulin sensitizer yields the insulin response which is followed by the desired lowering of blood glucose levels. Moreover, the reduction in blood glucose levels as observed with the composition according to the invention is markedly greater than what would be expected when either component is administered alone thus indicating a synergistic effect.

To obtain the composition with the most potent glucose levelling effect, it is desirable that the insulin sensitizer is suitably combined with an effective amount of a specific peptide fraction. Apart from efficacy, a high palatability, certain physicochemical aspects and low costs of the composition according to the invention are important aspects, especially if used in a preventive way.

Optimally suitable peptide fractions are characterized by a high content of small peptides rich in hydrophobic amino acid residues. These peptide fractions can be derived from proteins of either animal or plant origin. Examples of such proteins are milk proteins like whey or casein, meat proteins, egg proteins, soy proteins, wheat proteins, pea proteins, potato protein, lupine protein, rice proteins and maize proteins. Preferably the protein raw material is milk protein, soy protein, or maize protein or purified fractions thereof. In the present context, the term "peptide fraction" is understood to indicate that it may contain all types of peptides that may vary in length. The preferred peptide fraction has an average peptide chain length in the range of 2-40 amino acid residues (molecular weight ranging from 200- 4000 Daltons) and more preferably in the range of 2-20 amino acid residues. The average peptide chain can be determined using methods as specified in the Materials & Methods section.

The peptide fraction is preferably obtained by hydrolysing a suitable protein substrate. The protein raw material may be hydrolysed by one or more hydrolytic enzymes. The hydrolytic enzyme can be of animal, plant, yeast, bacterial or fungal origin. Preferably enzyme preparations are used that have a broad cleavage specificity and a low exo-peptidase activity to minimise the liberation of free amino acids. Preferred endoproteases with such characteristics are subtilisin (EC3.4.24.4 or Pescalase as supplied by DSM Food Specialities, Seclin, France or Alcalase as supplied by NOVO, Bagsvaerd, Denmark), thermolysin (EC3.4.24.4 or Thermoase as supplied by Daiwa Kasei, Osaka, Japan), neutral metallo protease (EC3.4.24.28 or Brewers Protease 2000 as supplied by DSM Food Specialities, Seclin, France or Neutrase as supplied by NOVO) or chymotrypsin (EC3.4.21.1). or papain (EC3.4.22.2). The peptide fraction that can be used to prepare a composition as disclosed in the present invention include all protein hydrolysates that can be obtained by enzymatic hydrolysis or chemical hydrolysis using common techniques as described in the literature and known to those skilled in the art.

To limit the level of bitter off-tastes that are usually generated upon the extensive hydrolysis of proteins, the peptide fraction is preferably obtained by incorporating a proline-

specific endoprotease. A proline specific endoprotease implies preferential cleavage at either the aminoterminal or the carboxyterminal side of proline. Endoproteases capable of cleaving at the aminoterminal side of proline are known (Nature, Vol 391, 15 January 15, pp301-304,1998). Endoproteases with a preference for cleaving at the carboxyterminal side of proline are also known (EC3.4.21.26). The latter type of proline-specific endoprotease is preferably obtained from food-grade overproducing recombinant strains such as *Aspergillus*. An example of a suitable producer of this enzyme has been described in WO 02/45523.

Most preferably the peptide fraction incorporated in the composition according to the invention is rich in di- and/or tripeptides. Rich in di and/or tripeptides means that at least 20 molar%, preferably at least 40molar%, more preferably at least 50 molar% of the peptides is present as di and/or tripeptides. As described in our copending patent application EP 02100667.1 the use of dipeptidyl- and/or tripeptidyl-peptidases in the production of hydrolysates is of special importance as these offer an efficient way for producing the peptide fraction according to the invention. Therefore preferably the peptide fraction is a hydrolysate preferably comprising a significant amount of peptides. Apart from obtaining the peptide fraction by protein hydrolysis, the peptide fraction can be obtained via chemical or enzymatic synthesis. Moreover a hydrolysate spiked with such synthesised peptides can form the peptide fraction as present in the composition according to the invention.

Preferably the peptide fraction is present in the composition according to the invention in an amount of 0.5-99 wt%, preferably 1.0-90 wt%, more preferably 1.5-50wt.%, calculated on dry matter basis of the composition.

By 'insulin sensitizing agent' or 'insulin sensitizer' is meant a compound that will lower blood glucose levels by increasing the responsiveness of the tissues to insulin. Examples of insulin sensitizing agents are trace elements like chromium, vanadium or a vitamin like niacin. Furthermore plant extracts from for example Banaba leaf have been shown to be effective insulin sensitisers and its active compound corosolic acid more in particular. Examples of medical "insulin sensitizers" are biguanides (such as Glucophage) and thiazolidinediones (such as Actos and Avandia).

The composition according the invention may contain a single insulin sensitising agent or combinations of such agents. Preferably the composition according to the invention contains these agents in their recommended daily dosages. The composition according to the invention may contain the peptide fraction and the insulin sensitiser in a mixed form or the peptide fraction and insuline sensitiser may be a separately packed and sold as a one package.

Apart from peptides and the insulin sensitising agent, the composition may optionally contain free amino acids and/or carbohydrates.

Especially free amino acids belonging to the group of leucine and/or arginine and/or phenylalanine and/or tyrosine are of importance. These free amino acids may be added to the peptide fraction to obtain the composition according to the invention. Because in some countries the addition of extra free amino acids is not allowed, the desired balance between the bound and free amino acids can be obtained using selective combinations of an endoprotease plus an exoprotease as outlined in WO02/32232. Preferred are compositions which are rich in amino acids from the group of leucine and/or arginine and/or phenylalanine and/or tyrosine.Preferably the free and peptide bound amino acids belonging to the group of leucine, arginine, phenylalanine and tyrosine are present in the composition of the invention in at least 10%wt, preferably 20%wt, more preferably 30%wt, calculated on dry weight, of the total of free and peptide bound amino acids Preferably the amino acids from this group are present in the composition in an amount of 0.599wt%, preferably 1.0-90wt%, more preferably 1.5-50wt%, calculated on dry matter basis of the composition.

The actual content of amino acids present in the final composition can be established using methods specified in the Materials & Methods section.

Apart from amino acids, carbohydrates are optionally present in the composition according to the invention. Depending upon the anticipated use, i.e. as such or in combination with other food, the composition may contain a separate source of carbohydrates. These carbohydrates can be glucose or more slowly absorbed carbohydrates like starch dependent upon the desired glycaemic- index for the particular application. In the present composition, carbohydrates can be present in an amount of 1.0-90% wt, preferably 2-50%wt, more preferably 6-35%wt.calculated on dry matter basis.

This wide range of carbohydrates can be explained on the anticipated use of the composition according to the invention.

On the one hand, an optimized formulation for consumers that prefer to take the composition alongside their carbohydrate containing meal or even prefer to take the composition in between meals. This group of consumers is likely to prefer an almost pure peptide + insulin sensitizer supplement, e.g. in the form of a tablet.

On the other hand consumers may prefer the composition that is integrated in a carbohydrate containing regular or "approved meal".

Other optional components of the composition according to the invention are vitamins, minerals, flavours, antioxidants, components having co-enzyme and antioxidant properties, lipids including emulsifiers, colourants, and proteins for meeting specific nutritional and/or physiological needs.

The composition of the present invention can be either a pharmaceutical composition or a food composition.

The composition according to the invention may have the form of a powder, a tablet, a capsule, other galenic forms, a beverage or any other food product.

The composition of the present invention may be part of a normal meal or part of an approved meal.

By "approved meal" or "approved diabetic diet" is meant a meal recommended or approved by a national nutritional organization for the health of a diabetic, for example, the American Diabetes Association Inc. ("ADA") as set forth, for example in "Maximizing the Role of Nutrition in Diabetes Management" published 1994 by the American Diabetes Association, Inc., the disclosures relating thereto being incorporated by reference thereto as if fully set forth herein, or an equivalent approval. Other Western organizations, which recommend or approve a meal for a diabetic, are The International Diabetes Federation; the European Association For the Study of Diabetes; and the European and Canadian Dietetic Association. Other Eastern and Far Eastern organizations are the Chinese Diabetes Federation; the Japanese Diabetes Federation; and the Indian Diabetes Federation. It will be appreciated that an "approved meal" will vary depending upon the

culture and geography of the diabetic, it being understood that, irrespective of either, a compliant diabetic will eat a meal, which makes no more than a reasonable demand upon his/her system.

Legends to the Figures

Fig. 1 Plasma insulin response (mU/ml/2h) for type 2 diabetes and a control group after drinking a beverage containing carbohydrate or carbohydrate/hydrolysate, respectively.

Fig.2 Plasma glucose response (mmol/l/2h) for type diabetes and a control group after drinking a beverage containing carbohydrate or carbohydrate/hydrolysate, respectively.

Materials and Methods

Sodium caseinate containing 90% protein was obtained from DMV International (The Netherlands). Subtilisin from B.licheniformis (Delvolase®, 560 000 DU per gram) was obtained from DSM Food Specialities (Seclin, France). Thermolysin (Thermoase; a heat stable metallo-endoprotease from Bacillus thermoproteolyticus Rokko with an activity of 14000 PU/ mg) was obtained from Daiwa Kasei, Osaka, Japan).
Whey protein was obtained as HIPROTAL 880 from Borculo Domò Ingredients (The Netherlands).

The enzymatic activity of proline specific endoproteases exhibiting pH optima above pH 6.0 are tested according to T.Diefenthal and H.Dargatz (World Journal of Microbiology &Biotechnology 11, 209-212 (1995)) on Z-Gly-Pro-pNA (Bachem Switzerland) 0.26 mM in phosphate buffer 0.1M pH 7.0 at 25°C. pH 7.0. The product was monitored spectrophotometrically at 410 nm.Proline specific endoproteases from Aspergillus was measured according to the method described in Japanese patent JP5015314 with minor modifications. In brief the enzymatic activity is tested on ZGly-Pro-pNA at 37 degrees C in a citrate/disodium phosphate buffer pH 5. pH 5.0 is chosen because in this test the pH optimum of the enzyme is below pH 6. The reaction product was also monitored spectrophotometrically at 410 nM. . The activity of the purified tripeptidyl aminopeptidase (TPAP) as over produced by A. niger was measured in a similar way. However, in this case the synthetic substrate Ala-Ala-Phe-pNA (Bachem, Switserland)

was used in an incubation in 0.1 mol/litre citrate buffer at pH 4.0 and 60 degrees C. The purified TPAP had an activity of 8 units/ml.
A unit is defined as the quantity of enzyme that provokes the release of 1 µmol of p nitroanilide per minute under these conditions.

The Degree of Hydrolysis (DH) as obtained during incubation with the various proteolytic mixtures was monitored using a rapid OPA test (JFS, Vol 66, NO 5, 2001).

Average peptide chain length in the various peptide fractions were determined by chromatography over a Superdex Peptide HR 1030 column.

Sensoric evaluation of the various peptide fractions was carried out by an independent institute availing of a panel trained in detecting and ranking various levels of bitterness. During the sssions the taste trials were performed 'blind' and bitterness was scored on a scale from 0 (none)- 4 (very bitter). Panel members were trained with quinine sulphate with the following solutions:

15 ppm quinine sulphate > Intensity bitter = 1
20 ppm quinine sulphate > Intensity bitter = 2
30 ppm quinine sulphate > Intensity bitter = 3
50 ppm quinine sulphate > Intensity bitter = 4

LC/MS analysis..

HPLC using an ion trap mass spectrometer (Thermoquest®, Breda, the Netherlands) coupled to a P4000 pump (Thermoquest®, Breda, the Netherlands) was used in characterising the enzymatic protein hydrolysates produced by the inventive enzyme mixture. The peptides formed were separated using a PEPMAP C18 300A (MIC-15-03-C18-PM, LC Packings, Amsterdam, The Netherlands) column in combination with a gradient of 0.1% formic acid + 1 mM nonafluoropentaoic acid (NFPA) in Milli Q water (Millipore, Bedford, MA, USA; Solution A) and 0.1% formic acid in acetonitrile (Solution B) for elution. The gradient started at 95% of Solution A and increased to 40% of solution B in 140 minutes and was kept at the latter ratio for another 5 minutes. The injection volume used was 50 microliters, the flow rate was 50 microliter per minute and the column temperature was maintained at 30°C. The protein concentration of the injected sample was approx. 50 micrograms/milliliter.

Detailed information on the individual peptides was obtained by using the "scan dependent" MS/MS algorithm which is a characteristic algorithm for an ion trap mass spectrometer.

Full scan analysis was followed by zoom scan analysis for the determination of the charge state of the most intense ion in the full scan mass range. Subsequent MS/MS analysis of the latter ion resulted in partial peptide sequence information, which could be used for database searching using the SEQUEST application from Xcalibur Bioworks (Thermoquest®, Breda, The Netherlands). Databanks used were extracted from the OWL.fasta databank, available at the NCBI (National Centre for Biotechnology informatics), containing the proteins of interest for the application used. In those experiments in which well characterized protein substrates such as whey proteins or caseins were measured, the precision of the analysis technique was increased by omitting those MS/MS spectra with a sequence fit of less than 50%.

By using different enzyme mixtures the mass range of the peptides formed starts at di- and tripeptides. By using the volatile ion-pairing reagent NFPA in combination with reversed phase liquid chromatography also smaller and more hydrophilic peptides can be monitored ending up with a mass ranging from approx. 200 to 2000 Daltons, considered suitable for further analysis by MS sequencing.

Angiotensin (M=1295.6) was used to tune for optimal sensitivity in MS mode and for optimal fragmentation in MS/MS mode, performing constant infusion of 60 mg/ml, resulting in mainly doubly and triply charged species in MS mode, and an optimal collision energy of about 35 % in MS/MS mode.

Determination of free amino acids in a hydrolysate which is completely hydrolyzed to free amino acids.

Amino acid analysis was carried out according to the PicoTag method as specified in the operators manual of the Amino Acid Analysis System of Waters (Milford MA, USA). Acid hydrolysis of the protein hydrolysates to obtain free amino acids, was achieved by vapour phase hydrolysis over 6 N HCl, also according to Waters. In brief this procedure is the following. A sample of the clear protein containing supernatant obtained after enzyme hydrolysis is homogenized in a dilute HCl solution. The resulting solution is then subjected to a vapour phase hydrolysis according to Waters. From the latter material a new sample

was taken, dried and derivatised using phenylisothiocyanate. The various derivatised amino acids present were quantitated using HPLC methods.

Since during acid hydrolysis Trp and Cys are destroyed, these amino acids are not included in the data presented. However, Gln and Asn residues are converted into Glu and Asp during acid hydrolysis so that the values for Glu and Gln, and for Asp and Asn were usually summed together to allow comparison with the data obtained before acid hydrolysis.

Examples

Example 1

Peptides and amino acids enhance plasma insulin levels but without diminishing high glucose levels in diabetic patients

The aim of this study was to investigate if a peptide fraction enriched with free leucine, phenylalanine could significantly lower blood glucose levels in type 2 diabetic patients.

Subjects

Ten (long-term) diagnosed male type 2 diabetic patients were selected to participate in this study. Exclusion criteria were impaired renal or liver function, obesity (BMI>30), cardiac disease, hypertension, diabetic complications and (exogenous) insulin therapy. Most subjects (n=8) were using oral antidiabetica (mainly sulfonylureas). Ten healthy, normoglycaemic, male adults, matched for age and body mass index (BMI), participated as controls. In the diabetic subjects blood glucose lowering medication was withheld for 3 days before participation in the trials and throughout the entire experimental period. Subjects were screened for glucose intolerance / type 2 diabetes by an oral glucose tolerance test (OGTT) according to the World Health Organisation criteria of 1999.

Study design

Each subject participated in 2 trials, separated by one week, in which the insulin response to the ingestion of 2 drink compositions (CHO or CHO+PRO) was determined. Both trials lasted 2 hours in which subjects were seated and remained inactive. Drinks were provided in randomised order and double blind.

Protocol

Subjects reported to the laboratory at 8:30 A.M., arriving by car or public transport, after an overnight fast. A Teflon catheter was inserted into an antecubital vein and a resting blood sample was drawn (t=0 minutes). Immediately thereafter subjects drank an initial bolus (3 $ml.kg^{-1}$) of a given test drink (CHO or CHO+PRO trial). Repeated boluses (3 $ml.kg^{-1}$) were taken every 30 minutes until t=90 minutes. Blood samples were drawn at 15 minutes intervals for measurement of plasma glucose and insulin concentrations.

Beverages

At t=0, t=30, t=60 and t=90 subjects received a beverage volume of 3 ml/kg to ensure a given dose of 0.8 g/kg/hour carbohydrate (50% glucose; 50% maltodextrin) with or without an additional 0.4 g/kg/hour of an amino acid-protein hydrolysate mixture (CHO+PRO or CHO trial, respectively). This hydrolysate/amino acid mixture consisted of a wheat protein hydrolysate (50%; 0.2 g/kg/hour), free leucine (25%; 0.1 g/kg/hour) and free phenylalanine (25%; 0.1 g/kg/hour).

Analysis

Blood (10 ml) was collected in EDTA containing tubes and centrifuged at 1000 g and 4 °C for 10 minutes. Aliquots of plasma were frozen immediately in liquid nitrogen and stored at –80 °C. Glucose (Unit Kit III, 07367204, Roche, Basel, Switzerland) was analysed with the COBAS FARA semi automatic analyser (Roche, Basel, Switzerland). Insulin was analysed by radio-immuno-assay (Insulin RIA 100 kit, Pharmacia, Sweden).

According to the results obtained (see figure underneath) the insulin responses of both the type 2 diabetic and control group were substantially increased by the addition of the CHO+PRO mixture. In the group consisting of type 2 diabetes insulin levels increased to 289% of the original 100% whereas in the reference group insulin levels increased to 214% of the original 100% ($P<0.01$). The results show that while keeping the glucose intake the same, addition of the peptide material thus results in a normalisation of the insulin response in type 2 diabetics i.e. the insulin levels become comparable with the levels reached by healthy persons ingesting glucose alone (see Figure 1). However, unlike the insulin response, plasma glucose concentrations were not differently affected between trials within this timeframe! (see Figure 2)

These observations lead to the surprising observation that although the insulin response in long-term diagnosed type 2 diabetic patients was substantially enhanced by the addition of the peptide material, no significant lowering effect was observed on their blood glucose concentrations.

Example 2

A non-bitter casein hydrolysate enriched in di-and tripeptides

Casein contains a high proportion of leucine, phenylalanine and tyrosine which makes it an excellent substrate for producing a peptide fraction according to the invention. However, due to this high content of hydrophobic amino acid residues, casein hydrolysates tend to be very bitter. Furthermore its high proline content prevent currently available endoproteases to hydrolyse this substrate to the level of di-and tripeptides. To circumvent these problems we have first incubated casein with a commonly available broad spectrum protease (i.e. Delvolase) and subsequently with a proline-specific endoprotease (EndoPro) to debitter the hydrolysate and to further increase the number of small peptides and finally with a tripeptidylaminopeptidese (TPAP) to increase the number of tripeptides in the hydrolysate.

A 6% (w/w on protein) casein solution was prepared by dissolving sodium caseinate in water. After adjustment of the pH to 8.0 by NaOH, the serine protease Delvolase was added to a concentration of 4% (v/v) and the mixture was incubated for 2.5 hours at 60 degrees C under non-pH-stat conditions. Then the reaction was stopped by lowering the pH to 5.0 using lactic acid followed by a heat treatment of 10 minutes at 90 degrees C. The solution was cooled down to 50 degrees C and two samples were taken. The first sample (Sample A) served as a reference characterizing the material that has been subjected to the action of a broad spectrum serine protease only. The second sample was used for subsequent incubations with EndoPro and finally TPAP. The incubation with EndoPro was carried out by adding a chromatographically purified

solution of the overproduced proline specific endoprotease from *A. niger* in a concentration of 2 units/ gram protein (see WO 02/45523). After incubating for 16 hours at 50 degrees C under non-pH-stat conditions the EndoPro enzyme was inactivated by another heat treatment to yield Sample B.

At this stage Samples A and B were sensorically evaluated by a trained panel. The two samples were tasted "blind" and then scored on a scale from 0 (non bitter) to 4 (very bitter) as described in the Materials & Methods section. Sample A was unanimously scored as "very bitter", Sample B was unanimously scored as "non bitter" hereby confirming the debittering capacity of the EndoPro enzyme.

Part of Sample B was then incubated with 20 units of chromatographically purified TPAP (see our copending patent application EP 02100667.1) per gram of casein protein during 5 hours at pH 4.0 and 60 degrees C. Like before the enzyme reaction was terminated by heating of the solution for 10 minutes at 95 degrees C to yield Sample C.

Samples A, B and C were then subjected to LC/MS analysis (see Materials & Methods section) to determine the size distribution of major peptides present. From all hydrolysates at least 124 different peptides were analysed. The data obtained are shown underneath.

Enzymes used to prepare casein hydrolysate	Heptapeptides or smaller (molar% of all peptides detected)	Di + tripeptides (molar% of all peptides detected)
Subtilisin ("Delvolase")	68	15
+ EndoPro (WO 02/45523)	65	17
+EndoPro+TPAP (EP 02100667.1)	76	21

Combining the results of the sensory evaluation and the LC/MS analysis, it is clear that an incubation with both EndoPro and TPAP (i.e. after an incubation with subtilisin) yields a superior product in terms of bitterness , allergenicity (peptides smaller than 8 amino acid residues) and its tripeptide content .

Example 3

A whey hydrolysate enriched in di- and tripeptides

Whey proteins contain a high proportion of leucine, phenylalanine and tyrosine and its hydrolysates are therefore well suited for preparing the peptide fraction according to the invention. Unlike caseins, whey contains low levels of proline residues only and whey hydrolysates tend to be non-bitter. In this example we will demonstrate the beneficial role of tripeptidylpeptidases in combination with a conventional broad spectrum protease to create hydrolysates with a high content in di-and tripeptides.

A 6% (w/w on protein) whey solution was prepared and treated with the broad spectrum endoprotease Delvolase as specified in Example 2. After lowering the pH to 4.5 using HCl Delvolase was inactivated by a heat treatment of 10 minutes at 95 degrees C. Then the solution was split in 2 halves, one half served as the reference and to the other half tripeptidylpeptidase (TPAP) was added in a concentration of 20 units/ gram protein. After incubation for 18 hours at 55 degrees C, the sample was heated for 5 minutes to 95 degrees C and frozen. To enable LC/MS analysis both samples were thawed, centrifugated, filtered through a 0.45 micron filter and diluted 5 times with water. Then LC/MS analysis was carried out as described in the Materials& Methods section.

In the sample treated with Delvolase only, 73 peptides were analysed of which 18 were found to be tripeptides. No dipeptides were found. So the whey hydrolysate prepared with Delvolase only contains a molar content of 25% di-and tripeptides in the water soluble fraction.

In the sample treated with Delvolase plus TPAP 79 peptides were analysed of which 31 were tripeptides and 2 were dipeptides. This represents a molar content of 42% of di-and tripeptides in the water soluble fraction.

Example 4

Peptides enriched with di- and tripeptides combined with an insulin sensitizer raise plasma insulin levels and diminish high glucose levels in diabetic patients

The aim of this study is to investigate if a peptide fraction enriched with di- and tripeptides combined with an 'insulin sensitizer' can raise the insulin response in type 2 diabetic patients, thereby lowering blood glucose levels.

Eight (long-term) diagnosed male type 2 diabetic patients all using oral 'insulin sensitizer' drugs (but no exogenous insulin) are selected to particpate in this study. Exclusion criteria are impaired renal or liver function, obesity (BMI>30), cardiac disease, hypertension, diabetic complications and (exogenous) insulin therapy.
Ten healthy, normoglycaemic, male adults, matched for age and body mass index (BMI), participate as controls.

Each subject participates in 2 trials, seperated by one week, in which the insulin and glucose response to the ingestion of 2 beverage compositions (Carbohydrate (CHO) or Carbohydrate + Peptide fraction according to the invention (CHO+PRO)) is determined while the diabetic patients maintain their usual 'insulin sensitizer' therapy. Both trials last 5 hours in which subjects are seated and are remained inactive. During this period a continuous intravenous infusion of [6,6-2H2]glucose is administered to determine plasma glucose disposal rates.
Drinks are provided in randomised order and double blind.

Protocol

Subjects report to the laboratory at 8:30 A.M., arriving by car or public transport, after an overnight fast. A Teflon catheter is inserted into an antecubital vein and a resting blood sample is drawn (t=0 minutes). Immediately thereafter subjects drink an initial bolus (3 ml.kg-1) of a given test drink (CHO or CHO+PRO trial). Repeated bolusses (3 ml.kg-1) are taken every 30 minutes. Blood samples are drawn at 15 minutes intervals for measurement of plasma glucose and insulin concentrations.

Analysis

Blood (10 ml) are collected in EDTA containing tubes and centrifuged at 1000 g and 4 degree Celsius for 10 minutes. Aliquots of plasma are frozen immediately in liquid nitrogen and stored at –80 degree Celsius. Glucose (Unit Kit III, 07367204, Roche, Basel, Switzerland) is analysed with the COBAS FARA semi automatic analyser (Roche,

Basel, Switzerland). Insulin is analysed by radio-immuno-assay (Insulin RIA 100 kit, Pharmacia, Sweden).

In contrast with the results recorded in Example 1, the combined use of insulin sensitizer therapy and a composition according to the invention results in an enhanced plasma insulin response plus a significantly lowered blood glucose level in type 2 diabetic patients.

CLAIMS

1. A composition comprising an insulin sensitizer and a peptide fraction.

2. A composition according to claim 1, which comprises an amount of at least one free amino acid, selected from the group consisting of leucine, phenylalanine and arginine.

3. A composition according to claim 1 or 2 whereby the peptide fraction is part of a hydrolysate.

4. A composition according to claim 2 whereby the free amino acid is part of a hydrolysate.

5. A composition according to any one of claims 1 to 4 whereby the peptide fraction has an average peptide chain length of 2-40 amino acids residues.

6 A composition according to any one of the claims 1 to 5 whereby the peptide fraction is rich in di and/or tripeptides preferably whereby the di-and/or tripeptides are rich in proline at one end of the peptide.

7 A composition according to claim 6 wherein at least 20molar%, preferably at least 25molar%, more preferably at least 30molar% of the peptide fraction is present as di and/or tripeptide.

8 A composition according to claim 6 or 7 wherein at least 20%, preferably at least 30%, more preferably at least 40% of the proline present in the starting protein is present in the di and/or tripeptides.

9 A composition according to any one of claims 6 to 8 wherein 30% of the tripeptides, preferably 35% of the tripeptide have a carboxy terminal proline.

10 A composition according to any one of claims 6 to 9 wherein at least 70 molar% of the peptides, preferably at least 75 molar% of the peptides contain 2 to 7 amino acid residues (dipeptide to heptapeptide).

11. A dietic product or a pharmaceutical or a food or a food supplement comprising the composition according to any one of claims 1 to 10.

12. Use of a composition according to any one of claims 1 to 10 from human consumption.

13. Use of a composition comprising a peptide fraction for diabetes types 2.

14. Use of a composition comprising a peptide fraction and at least one free amino acid selected from tyrosine, leucine, phenylamine and arginine for diabetes Type 2.

ABSTRACT

The present invention discloses a composition comprising an insulin sensitizer and a peptide fraction, which promotes significant reduction in blood glucose levels and stabilizes blood glucose levels in individuals with type 2 diabetes.

FIGURES

Fig. 1

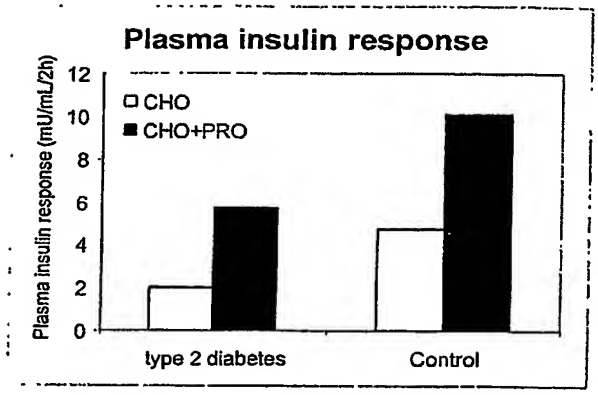
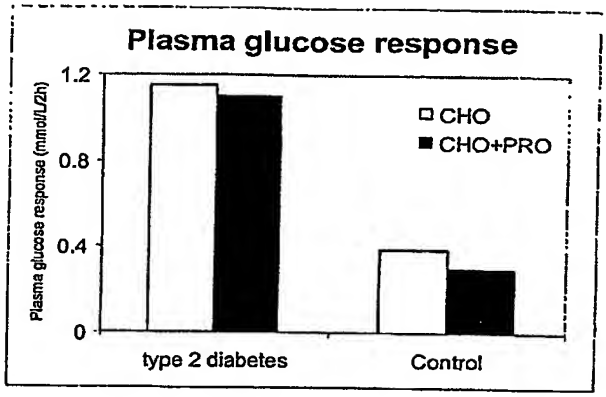


Fig.2



This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☑ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.